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Model Systems for Studying the Role of Canalicular Efflux Transporters in Drug-induced Cholestatic Liver Disease

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Abstract

Bile formation is a key function of the liver. Disturbance of bile flow may lead to liver disease and is called cholestasis. Cholestasis may be inherited such as for example in progressive familial intrahepatic cholestasis or acquired, such as for example by drug-mediated inhibition of bile salt export from hepatocytes into the canaliculi. The key transport system for exporting bile salts into the canaliculi is the bile salt export pump. Inhibition of the bile salt export pump by drugs is a well-established cause of drug-induced cholestasis. Investigation of the role of the multidrug resistance protein 3, essential for biliary phospholipid secretion, is emerging now. This overview summarizes current concepts and methods with an emphasis on in vitro model systems for the investigation of drug-induced cholestasis in the general context of drug-induced liver injury.

Abbreviations:

BSEP, bile salt export pump; DILI, drug-induced liver injury; FXR, farnesoid X receptor; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; NTCP, sodium taurocholate-cotransporting polypeptide; OATP, organic anion transporting polypeptide; OST, organic solute transporter; PFIC, progressive familial intrahepatic cholestasis.

Introduction

Bile formation is an important function of the liver. Bile assists fat digestion in the intestine and mediates the disposal of poorly water soluble endogenous compounds and xenobiotics. The major constituents of bile are bile salts (in humans about 67 %), which are taurine or glycine conjugates of bile acids and lipids (in humans about 27 %) [1]. Canalicular bile is produced by hepatocytes, which secrete solutes into the canaliculi followed by water in an isosmotic process [2]. Hepatocellular bile formation requires the coordinate functioning of basolateral solute uptake systems and canalicular export systems for solutes [3]. This canalicular bile is modified in the bile ducts by cholangiocytes and thereafter leaves the liver [3]. Bile flow can be separated into bile salt dependent bile flow, which is driven by canalicular bile salt secretion and bile salt-independent bile flow, which is driven by canalicular secretion of organic anions (other than bile salts) and by bicarbonate secretion into the canaliculus. In addition, cholangiocytes contribute significantly to bile salt-independent bile flow by secreting bicarbonate into bile [3]. In liver disease, bile flow may be impaired to different degrees, a condition which was named cholestasis more than 50 years ago [4].

Hepatocellular bile salt transport systems

Bile acids and bile salts reach the liver from the intestine via the portal vein and are taken up into hepatocytes across the basolateral membrane, which is exposed to blood plasma. In order to efficiently transport bile salts from the portal blood into bile, hepatocytes express several uptake and efflux systems in a polarized manner (figure 1).

Under undisturbed physiologic conditions, bile salts are predominantly absorbed by the basolateral sodium taurocholate cotransporting polypeptide (NTCP, *SLC10A1* [protein name, *gene name*]). As this process is electrogenic, transport may occur against a concentration gradient [5,6]. Sodium-independent uptake of bile acids is mediated by organic anion transporting polypeptides (OATP1B1, *SCLO1B1*, OATP1B3, *SLCO1B3* and OATP2B1, *SLCO2B1*). In vitro experiments have shown that hepatocellular OATPs can mediate transport of unconjugated bile acids and conjugated bile salts [7]. Once in hepatocytes, bile salts reach by a poorly understood mechanism the canalicular membrane. In hepatocytes, imported and newly synthesized bile acids are rapidly and efficiently conjugated to taurine or glycine [8,9]. Export of bile salts across the canalicular membrane occurs against a steep concentration gradient and is mediated by a member of the ATP-binding cassette (ABC) transporter superfamily. The exclusive transporter for canalicular secretion of primary bile salts is the bile salt export pump (BSEP, *ABCB11*) [5]. The release of phosphatidylcholine (PC), the major phospholipid in human bile [10,11], into the canaliculus requires at the canalicular membrane the activity of the PC translocator MDR3 (*ABCB4*) and the luminal detergent action of bile salts secreted into the canaliculus [12]. The P4-ATPase ATP8B1 (*ATBP8B1*) is an aminophospholipid translocator, which assists in maintaining the integrity of the lipid bilayer of the canalicular membrane [13]. The physiologic role of additional P4-ATPases expressed at the canalicular membrane is currently unknown [14]. The multidrug resistance-associated protein 2 (MRP2, *ABCC2*) exports bilirubin conjugates as well as other mainly glucuronidated metabolites of xenobiotics [15]. ABCG2 (*ABCG2*) mediates the canalicular excretion of a large chemical variety of xenobiotics including xenobiotic conjugates [16]. This set of transporters is complemented by the multidrug resistance protein 1 (MDR1, *ABCB1*), which mediates canalicular export of a wide variety of structurally rather different compounds [17].

In addition to the above mentioned transport systems, hepatocytes express at the basolateral membrane additional efflux systems (figure 1). MRP3 (*ABCC3*) seems to be a low capacity bile salt transporter transporting glycocholate but not taurocholate [18]. However, it transports

the mono- and bisglucuronide conjugates of bilirubin [19] and drugs and drug metabolites [20]. MRP4 acts as a glutathione taurocholate cotransporter [21] and transports a wide variety of endogenous and exogenous compounds [22]. The heterodimeric OSTα/OSTβ is another transport system capable of mediating bile salt efflux and other steroid-derived molecules [23]. Of note, the exact transport mechanism of this transporters remains somewhat elusive, while it is established than an association of the two subunits is required for targeting the transporter after biosynthesis to the plasma membrane [24].

Cholestasis

Cholestasis, originally defined as impaired bile flow [4], comprises a variety of clinical entities [25] with common clinical features, such as elevated serum alkaline phosphatase and in severe cases jaundice.

Cholestasis may be inherited, such as for example in the case of progressive familial intrahepatic cholestasis (PFIC), a severe liver disease often requiring liver transplantation at young age [26]. PFIC1, PFIC2 and PFIC3 are caused by mutations in *ATP8B1*, *ABCB11* and *ABCB4*, respectively [27]. The identification of mutations in these genes was instrumental for demonstrating the key role of these ATP-dependent systems in canalicular bile formation and in bile salt homeostasis in hepatocytes [28,29]. As neither BSEP nor MDR3 (and ATP8B1) have functional backup systems, any persistent impairment of these transporters will ultimately lead to liver disease. Recently, a novel form of PFIC has been described, which is due to mutations in the *NR1H4* gene encoding the farnesoid X receptor (FXR) [30]. FXR is a bile acid sensor, which protects hepatocytes from increased bile salt concentrations by repressing the biosynthesis of bile acids, repressing the uptake of bile salts and by increasing the canalicular export of bile salts via BSEP [31]. Four patients with non-functional FXR from two unrelated families did not express BSEP and developed severe PFIC leading to death at 5 weeks and 8 months, respectively or requiring liver transplantation earlier than two years of age [30]. This highlights the critical role of FXR for BSEP expression in human liver and for the proper bile salt homeostasis in hepatocytes.

Alternately, cholestatic liver disease can also be acquired, e.g. as intrahepatic cholestasis of pregnancy, inflammation-induced cholestasis (during sepsis), drug-induced cholestasis, primary biliary cirrhosis or as primary sclerosing cholangitis [32]. While PFICs are rare, monogenetic diseases, acquired forms of liver disease are more frequent and are caused by

multiple intrinsic and exogenous factors and may include a genetic predisposition, i.e. polymorphic variants of susceptibility genes [33,34]. The understanding of the molecular pathogenesis of acquired cholestatic liver diseases is incomplete and the currently available different animal models (e.g. for primary cholangitis) may not complete model such forms of liver disease [6,35-38].

Drug-induced cholestatic liver disease is a subtype of drug-induced liver injury (DILI) [39,40]. Idiosyncratic DILI is difficult to diagnose and is in essence a diagnosis by exclusion [41]. The incidence of DILI is reported as a wide range of figures [42] and may indeed be underdiagnosed [43]. DILI can be classified in a hepatocellular pattern, a cholestatic pattern and a mixed pattern based on specific changes of serum alkaline phosphatase and aminotransferase levels [39,44]. The incidence of cholestatic and mixed DILI is not known, but my account for up to 50 % of DILI [42,45].

Role of BSEP in drug-induced cholestasis

As BSEP is the only hepatocellular export system for primary bile salts into the canaliculus, interference of drugs and/or their metabolites with BSEP function may lead to drug-induced cholestasis. Such a mechanism has been reported for bosentan [46] and is now a well-accepted mechanism for drug-induced bland cholestasis [43]. BSEP may be inhibited directly from the cis-side (cytoplasm) by drugs and/or drug metabolites [47,48]. In addition, as exemplified by estradiol-17β-glucuronide, BSEP may be inhibited from the trans-side (canaliculus), a process which requires secretion of the perpetrator into the canaliculus prior to its inhibitory action [18,47,48]. Using a vesicular transport assay with BSEP expressed in insect cells [48], over 600 drugs have been tested for their potential as BSEP inhibitors [4948]. Morgan and coworkers found a 95 % association of 109 drugs with different forms of DILI after factoring the IC_{50} values for BSEP inhibition with drug exposure. This clearly demonstrates the validity and predictive potential for the involvement of BSEP in DILI. When in addition the inhibitory potential of these drugs against MRP2, MRP3 and MRP4 was taken into account, the correlation with different forms of DILI was close to complete [49].

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In addition to directly or indirectly inhibiting the transport activity of BSEP, drugs may also affect the transcriptional regulation of *ABCB11* and consequently BSEP expression leading to DILI. In a primary human hepatocyte culture system, out of 30 drugs known to inhibit the

transport activity of BSEP, five drugs including troglitazone and lopinavir were found to be potent repressors of *ABCB11* based on mRNA and protein determination [54]. In vitro trans-activation assays showed a concentration-dependent inhibition of FXR activity and hence these drugs can be considered as FXR antagonists [54]. Following a different approach, a study using mechanistic modeling of bile salt homeostasis in the presence of a theoretical BSEP inhibitor revealed that the most important unknowns of bile salt homeostasis, based on a sensitivity analysis, are the level of BSEP expression, the magnitude of FXR-regulation and the intestinal production of the secondary bile acid lithocholic acid [55]. With a system pharmacology approach in combination with in vitro studies, some non-steroidal anti-inflammatory drugs were also found to be FXR antagonists [56] These studies suggest a role of FXR in DILI, however the relevance/and or importance of FXR in DILI are not yet worked out in detail [57]. Adding an additional level of regulation (and complexity) to bile salt homeostasis, the micro RNA miR-33 has been found to downregulate the mRNAs for mBSEP and mATP8B1 in a mouse model [58]. In the same model, statins can induce miR-33, which in turn leads to a down-regulation of mRNAs coding for mBSEP and mATP8B1. Such a mechanism may contribute to statin-induced liver injury. However, it should be noted that the statin concentrations used in the above experiments were very high.

It should be kept in mind that not only parent compounds but also drug metabolites can inhibit efflux systems, as e.g. demonstrated for bosentan [46] or troglitazone-sulfate and BSEP [50]. The mechanism of troglitazone-induced DILI is complex and may also involve damage of mitochondria by reactive metabolites [48,51]. Inhibition of BSEP by troglitazone and troglitazone-sulfate leads to an intracellular accumulation of bile salts, which are toxic to mitochondria [52]. Given the antagonistic effect of troglitazone (and other drugs) on FXR, the hepatoprotective response triggered by FXR activation through elevated bile salts [59] may well be attenuated. Such a process could further aggravate hepatocyte injury and consequently result in severe liver injury. The example of troglitazone shows that the establishment of the exact mechanism of DILI requires different and complementary experimental models. Hence, using a transport assay with membrane vesicles overexpressing BSEP is just one of several tools for investigating the role of BSEP (and other transporters) in cholestatic liver injury [53].

Role of MDR3 in drug-induced cholestasis

Considering that mutations in *ABCB4* coding for MDR3 lead to severe liver disease, it is conceivable that inhibition of this ABC-transporter by drugs would also lead to drug-induced

acquired liver injury. One can speculate that, similarly to patients with mutations in *ABCB4* [26], serum levels of γ-glutamyl transpeptidase (γGTP) would be elevated in patients with drug-mediated inhibition of MDR3. Indeed, three patients with DILI receiving itraconazole were reported to have DILI with elevated γGTP [60]. In an in vitro secretion assay based on biosynthetically labeling PC with [14C]choline, 1 μM itraconazole was found to inhibit MDR3 activity by about 50 % [60]. Using a deuterated choline as PC precursor and primary human hepatocytes kept in suspension, an IC_{50} of 2.1 μM for itraconazole on the inhibition of PC efflux from human hepatocytes was observed [61]. As canalicular lipid secretion requires the interaction of three different ABC transporters [12], a polar LLC-PK1 cell line stably expressing BSEP, MDR3 and ABCG5/ABCG8 at the apical membrane and NTCP at the basolateral membrane was established [62]. Using this cell line for modelling canalicular lipid secretion, itraconazole was found to inhibit the secretion of the fluorescent PC derivative 1-palmitoyl-2-{6-[(7-nitro-2-1,3-bonzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phoshpocholine (C_6-NBD-PC) by 42 % at 10 μM.

Hence, while all three mentioned studies found qualitatively an inhibition of MDR3 by itraconazole, there were considerable quantitative differences. All three research groups used fundamentally different experimental systems. Yoshikado and coworkers incubated a transiently transfected cell line with radioactively labelled choline as a biosynthetic precursor for PC and determined the PC efflux in the presence of extracellular 3 mM taurocholate. It is interesting to note that taurocholate stimulated PC secretion by 100 % in MDR3 transfected cells in comparison to GFP transfected cells, which already displayed a considerable PC efflux [60]. The latter could indicate that taurocholate impaired the viability of the cells. He and coworkers measured the release of a biosynthetically labelled PC from suspended human hepatocytes at an unspecified extracellular taurocholate concentration [61]. Hepatocytes in suspension very likely show no cell polarity with respect to the expression of ABC-transporters [63], similar to what is observed for freshly thawed hepatocytes suspensions [64]. Both of the above mentioned studies did also not use an acceptor for the poorly water soluble PC besides taurocholate and did not provide information, whether the impact of taurocholate on cell viability was checked. In both studies, an interference of itraconazole (and other drugs tested) on the biosynthesis of PC cannot entirely be ruled out. Mahdi and coworkers tested the impact of itraconazole on MDR3 mediated secretion of the fluorescent PC C_6-NBD-PC under a basolateral to apical, i.e. transcellular flux of taurocholate, which mimics the physiologic situation in hepatocytes and with albumin in the apical compartment as phospholipid acceptor

[62]. To the best of our knowledge, currently no data on the affinity (K_m) of PC or C6-NBD-PC to MDR3 are available. However, since the chemical structures of C_6-NBD-PC and PC differ, also their K_m values for MDR3 might differ. This difference may explain the lower potential of itraconazole for MDR3 inhibition in the study by Mahdi et al., which observed the following rank order for MDR3 inhibition by different azoles: posaconazole > itraconazole > ketoconazole, while fluconazole and voriconazole did not inhibit. He et al. observed the following rank order for azoles: itraconazole (I_{C50} 2.1 μM) > clotrimazole (IC_{50} 4.6 μM) > ketoconazole (IC_{50} 5.6 μM). Using a transcellular taurocholate transport assay in their novel cell line, Mahdi et al could demonstrate that itraconazole ~ posaconazole < ketoconazole inhibit apical taurocholate secretion by inhibiting BSEP. This rank order parallels published IC_{50} values as BSEP inhibitors for these drugs: 18 μM, 8.1 μM and 3.3 μM, respectively [65].

*Impact of expression system on transporter function and inhibition constants*Taking these three studies on the inhibition of MDR3 by drugs together, it is evident that the different conditions and experimental systems used lead to the quantitatively different findings. Hence, for becoming a valuable predictive tool, systems and assays for the assessment of the inhibition of MDR3 by drugs will certainly need a standardization. A similar approach is also needed for studying the in vitro the interaction of drugs with BSEP. The v_{max} of BSEP is known to be sensitive to membrane cholesterol content [66,67]. In addition, it has been demonstrated that the cooperativity of MRP2 may change in a substrate-dependent manner depending on the cholesterol content of the membrane expression system [67]. These examples clearly demonstrate a need for standardization of models used for in vitro assessment of transporter-related DILI, both with respect to cell system used as well as with respect to detailed assay conditions. This necessity can be further illustrated by a study involving 23 laboratories and a set of 16 inhibitors of MDR1, which yielded a 796-fold maximal range for an IC_{50} value between the involved laboratories [68]. Such differences approaching three orders of magnitude of variability for kinetic transporter parameters are clearly not useful for any predictions and extrapolations from an in vitro to an in vivo situation. The necessary standardization of assays in order to make results between different laboratories better comparable, e.g. in the area of transport studies, is only at the beginning [69]. Importantly, it has to be kept in mind that determination of IC_{50} values for drugs interacting with transporters does not provide information on the type of inhibition, which however needs to be known for extrapolation to the in vivo situation. Lastly, in vitro results should also be seen in the context of the interindividual variability of transport protein expression in healthy human tissues, e.g.

human liver. For example, studies using different methodologies showed in what was defined as healthy human liver tissue an expression variability for BSEP of 20-fold [70] or 4-fold [71], for MDR3 37-fold [70] and 8-fold [71] and for MDR1 22-fold [70], 2-fold [71] or 8-fold [72]. Part of these differences may be related to differences in liver tissue used and in technical issues, but it is conceivable that the interindividual variability of transporter expression is considerable between different individuals [73]. Such differences in protein expression levels between individuals will certainly contribute as a susceptibility factor for drug induced cholestatic liver injury and are currently not reflected in the model systems used to assess cholestatic DILI.

Model systems for in vitro investigation of drug-induced cholestasis

There is clearly a need for using different model systems for assessing the potential for drug-induced cholestasis of new chemical entities, as different expression systems may lead to different quantitative and/or qualitative findings (see above). An overview of examples of different experimental systems currently in use for the investigation of the inhibition of e.g. BSEP or other efflux transporters is listed in table 1.

In clinics, diagnosis of drug-induced cholestasis or DILI is very challenging [74]. However, a careful clinical description of such patients may lead to a detailed hypothesis on the involved molecular mechanism of the pathophysiology and as such, patients experiencing DILI should be considered a truly "holistic model" of DILI. The precision of the prediction of a mechanism from patients may be improved by using evolving new DILI biomarkers for plasma [75] and/or urine [76]. It is likely that non-invasive imaging of bile flow in patients with suspected drug-induced cholestasis may soon be possible [77]. Postulated mechanisms can subsequently be tested in animal models, such as e.g. isolated perfused rodent livers. Such models offer the advantage of representing the entire complexity of a liver including different cell types and regulatory mechanism. It should however be kept in mind that drug metabolism (e.g. metabolites produced) may differ between rodents and humans. Liver slices and primary cultured hepatocytes from human or animal tissues offer a less complex model than the intact organ [78]. These systems can likely be tested at hepatocellular bile salt concentrations close to the in vivo concentration, but suffer from dedifferentiation processes leading to an altered expression of transport proteins and drug metabolizing enzymes [78]. Finally, a specific molecular hypothesis (e.g. BSEP inhibition) can be tested in cell culture systems expressing transporters [47] or drug-metabolizing enzymes [79,80]. Simultaneous expression of phase I and phase II enzymes together with a drug-metabolite transporter has been reported for MRP2 [81] and should also be feasible for BSEP. The investigation of mechanisms of DILI will always

be a balance between easily to be used systems and more labor-intense complex holistic models [82].

Outlook

Finally, for successfully predicting (idiosyncratic) DILI, which results from the interplay of many different factors [83], "complex" models integrating a multitude (or ideally all) factors and different liver cell types would be needed. Development of liver organoids is an emerging research area [84,85]. An alternate approach, which is currently pursued is represented by so called "organ-on-a-chip" models [86-88]. Such approaches might be combined or paralleled by in silico approaches [89,90]. While physiologically based pharmacokinetic (PBPK) modelling approaches involve transport and metabolism [91,92] and are combined with wetlab approaches (e.g. drug sulfonation [93]), it might be interesting to further evolve such models by taking into account a possible specific special arrangement of transporters and drug-metabolizing enzymes in hepatocytes. The role of such arrangements is well appreciated in energy metabolism and is termed metabolic channeling [94,95]. A complementary in silico approach constitutes the development of predictive models based on physicochemical descriptors for drug-transporter interaction, as recently exemplified for BSEP [96]. Even though considerable progress has been made in developing test systems for (cholestatic) DILI, in particular for elucidating the intrinsic toxicity of a drug, developing models for studying idiosyncratic toxicity remains challenging to date [97]. It should however also be kept in mind that so far no in vitro cell system modelling the liver and capable of generating bile flow is available and it is likely that such an achievement will be very challenging. At the clinical level, search of susceptibility factors for DILI using genome-wide association studies with DILI [98] has lead e.g. to the identification of HLA class I and II alleles as susceptibility factors [99,100] as well as to the identification of genes coding for drug-metabolizing enzymes [101]. Genetic association studies however have not always been successful [102]. Induced pluripotent stem cells from thoroughly characterized patients suffering from DILI might become an important tool, as they carry the (complex) genotypes potentially contributing to DILI in the specific situation [103,104]. Such cells might well be used as source for use in a classical setup in vitro setup or in novel in vitro test systems aimed at predicting DILI. Additionally, and importantly, there is a need for novel and more sensitive markers for positively identifying DILI in daily clinical practice [105]. Finally, novel methods, like imaging are rapidly induced and allow visualization of drug transport in humans [106] and are reviewed in [107-110].

Table 1

Model systems used for studying drug-induced cholestasis

level of complexity	**endpoint**	**assay system**	**comment**	**Reference** (examples)
organism	global liver function	liver enzymes serum bile salt levels ICG clearance	amenable to humans (retrospective) and animals indirect evidence for BSEP involvement	[111,112]
organ	global liver function	imaging methodologies	Imaging is emerging and amenable to humans	[107,113,114]
	bile formation	in situ and isolated perfused liver and	quantitative data on bile flow and bile composition. secretion of metabolites back into plasma can be determined	[115,116]
		liver slices	3D-organization remains in vitro technically challenging, limited life span quantification of substances in bile is difficult	[117]
organoid	hepatotoxicity	spheroids	Allows co-culture of different liver cell types, assessment of transport processes only indirectly possible	[84,85]

cell	specific functions	primary hepatocytes in suspension; plated hepatocytes	Presence of multiple transporters, but state of differentiation not constant; lack of different liver cell types inter donor variability can be advantage or disadvantage interplay of metabolism and transport present determination of bile salt secretion rather indirect	[78]
		sandwich cultured hepatocytes	increased stability of differentiation over extended culture period interplay of metabolism and transport present assessment of canalicular secretion possible	[118]
subcellular level	specific functions	isolated canalicular vesicles	substrate binding site experimentally accessible assays with cytotoxic compounds possible interplay of transporters can be assessed	[47]

			metabolism is lacking	
individual transporter	direct characterization	transfected polarized cell lines	determination of transcellular fluxes for measuring efflux control for endogenous transporters necessary requires cotransfection with uptake systems cotransfection with efflux systems and metabolizing enzymes possible	[62,81,119,120]
		isolated membrane vesicles from transfected nonpolar cell lines	substrate binding site available assays with cytotoxic compounds possible cotransfection with efflux systems possible membrane lipid compositon between insect cells and mammalian cells differs	[47,49,67]

Legend to figure 1: **Key transporters in human hepatocytes**

Transporters at canalicular membrane in the center between two schematic hepatocytes are exporting substrates into bile. Transporters at the basolateral membrane facing the portal blood are importing substances into hepatocytes or exporting substances from hepatocytes into the portal blood. NTCP mediates bile salt uptake, OATPs, mediated uptake of bile salts and numerous drugs. BSEP exports bile salts into the canaliculus. MRP2 and ABCG2 mediate export of negatively charged metabolites of endogenous and exogenous compounds into the canaliculus. MDR1 mediates neutral and positively charged xenobiotics into the canaliculus. ABCg5/ABCG8, ATP8B1 and MDR3 translocate lipids in the canalicular membrane. MRP3, MRP4 and Ostα/β export endogenous and exogenous compounds into the portal blood.

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Table 1

Model systems used for studying drug-induced cholestasis

level of complexity	**endpoint**	**assay system**	**comment**	**Reference** (examples)
organism	global liver function	liver enzymes serum bile salt levels ICG clearance	amenable to humans (retrospective) and animals indirect evidence for BSEP involvement	[111,112]
organ	global liver function	imaging methodologies	Imaging is emerging and amenable to humans	[107,113,114]
	bile formation	in situ and isolated perfused liver and	quantitative data on bile flow and bile composition. secretion of metabolites back into plasma can be determined	[115,116]
		liver slices	3D-organization remains in vitro technically challenging, limited life span quantification of substances in bile is difficult	[117]
organoid	hepatotoxicity	spheroids	Allows co-culture of different liver cell types, assessment of transport processes only indirectly possible	[84,85]

cell	specific functions	primary hepatocytes in suspension; plated hepatocytes	Presence of multiple transporters, but state of differentiation not constant; lack of different liver cell types inter donor variability can be advantage or disadvantage interplay of metabolism and transport present determination of bile salt secretion rather indirect	[78]
		sandwich cultured hepatocytes	increased stability of differentiation over extended culture period interplay of metabolism and transport present assessment of canalicular secretion possible	[118]
subcellular level	specific functions	isolated canalicular vesicles	substrate binding site experimentally accessible assays with cytotoxic compounds possible interplay of transporters can be assessed	[47]

			metabolism is lacking	
individual transporter	direct characterization	transfected polarized cell lines	determination of transcellular fluxes for measuring efflux control for endogenous transporters necessary requires cotransfection with uptake systems cotransfection with efflux systems and metabolizing enzymes possible	[62,81,119,120]
		isolated membrane vesicles from transfected nonpolar cell lines	substrate binding site available assays with cytotoxic compounds possible cotransfection with efflux systems possible membrane lipid compositon between insect cells and mammalian cells differs	[47,49,67]